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A PEST-like element in FREQUENCY determines the length of the circadian period in *Neurospora crassa*

Margit Görl, Martha Merrow¹,
Benedikt Huttner, Judy Johnson¹,
Till Roenneberg¹ and Michael Brunner²

Biochemie-Zentrum Heidelberg, Im Neuenheimer Feld 328,
D-69120 Heidelberg and ¹Institut für Medizinische Psychologie und
Immunologie, Universität München, Goethestrasse 33,
D-80336 Munich, Germany

²Corresponding author

e-mail: Michael.Brunner@urz.uni-heidelberg.de

FREQUENCY (FRQ) is a crucial element of the circadian clock in *Neurospora crassa*. In the course of a circadian day FRQ is successively phosphorylated and degraded. Here we report that two PEST-like elements in FRQ, PEST-1 and PEST-2, are phosphorylated *in vitro* by recombinant CK-1a and CK-1b, two newly identified *Neurospora* homologs of casein kinase 1 ϵ . CK-1a is localized in the cytosol and the nuclei of *Neurospora* and it is in a complex with FRQ *in vivo*. Deletion of PEST-1 results in hypophosphorylation of FRQ and causes significantly increased protein stability. A strain harboring the mutant *frq* Δ PEST-1 gene shows no rhythmic conidiation. Despite the lack of overt rhythmicity, *frq* Δ PEST-1 RNA and FRQ Δ PEST-1 protein are rhythmically expressed and oscillate in constant darkness with a circadian period of 28 h. Thus, by deletion of PEST-1 the circadian period is lengthened and overt rhythmicity is dissociated from molecular oscillations of clock components.

Keywords: casein kinase 1 ϵ /circadian/frequency/
Neurospora/PEST

Introduction

Most organisms use circadian clocks to regulate physiological and behavioral activities in a time-of-day specific manner. Circadian clocks are cell-autonomous and mediate rhythmic expression of a broad variety of clock-controlled genes with a periodicity of approximately one day even in the absence of environmental cues. Circadian clocks are entrainable by exogenous stimuli (zeitgebers) such as light, temperature and nutrition. Entrainment synchronizes the endogenous oscillations with the exogenous time and allows behavioral flexibility. On the molecular level circadian oscillations are supported by interconnected transcriptional–translational feedback loops of considerable complexity. The interconnected loops seem to function in a similar manner in fungi, flies and mammals (Dunlap *et al.*, 1999; Young, 2000; Albrecht, 2001; Reppert and Weaver, 2001; Williams and Sehgal, 2001).

The circadian system of *Drosophila melanogaster* is well characterized on the molecular level. *Drosophila* CLOCK (CLK) and CYCLE (CYC) are Per-Arnt-Sim-like (PAS) domain-containing transcription factors that control transcription of *period* (*per*) and *timeless* (*tim*) RNA (Allada *et al.*, 1998; Darlington *et al.*, 1998; Rutila *et al.*, 1998; McDonald *et al.*, 2001; Wang *et al.*, 2001). When PER and TIM proteins are synthesized they form a complex that enters the nucleus and represses transcription of their own genes by inactivating CLK–CYC (Hardin *et al.*, 1990; Saez and Young, 1996; Lee *et al.*, 1998, 1999). During the course of a day PER and TIM are degraded, which again allows synthesis of *per* and *tim* RNA by CLK–CYC (Young, 2000; Williams and Sehgal, 2001). The length of a circadian period is affected by the kinetics of nuclear entry and degradation of PER–TIM. TIM is phosphorylated by SHAGGY, which regulates nuclear translocation of the PER–TIM heterodimer (Martinek *et al.*, 2001). The casein kinase 1 ϵ (CK-1 ϵ) homolog DOUBLETIME (DBT) triggers degradation of PER via phosphorylation and thereby regulates period length (Price *et al.*, 1998; Rothenfluh *et al.*, 2000; Kloss *et al.*, 2001). PER and TIM not only repress synthesis of their own genes but also support the expression of CLK (Bae *et al.*, 1998; Glossop *et al.*, 1999). Furthermore, TIM increases *per* RNA levels through a post-transcriptional mechanism (Suri *et al.*, 1999).

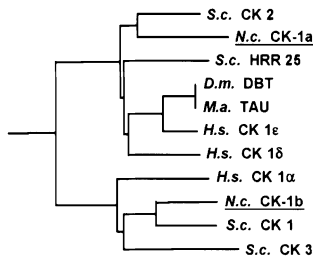
A central element of the circadian feedback loop of *Neurospora crassa* is *frequency* (*frq*). Expression of *frq* RNA depends on the oligomeric PAS domain containing transcription factors white collar (WC)-1 and WC-2 (Crosthwaite *et al.*, 1997; Talora *et al.*, 1999). On the other hand, FRQ controls the expression of *wc-1* RNA (Merrow *et al.*, 2001) and WC-1 (Lee *et al.*, 2000; Cheng *et al.*, 2001). As a consequence *frq* RNA and FRQ protein levels oscillate under free-running conditions (constant darkness) with a period of ~22 h (Aronson *et al.*, 1994; Garceau *et al.*, 1997). *frq* RNA levels reach a maximum in the subjective late morning followed by a FRQ protein peak ~4 h later (Garceau *et al.*, 1997; Merrow *et al.*, 1999). FRQ protein enters the nucleus and, when expressed at sufficiently high levels, represses synthesis of its own RNA (Aronson *et al.*, 1994; Merrow *et al.*, 1997; Luo *et al.*, 1998).

In the course of the day, FRQ protein is progressively hyperphosphorylated and then degraded. When FRQ protein levels fall below a threshold *frq* RNA is synthesized again and a new circadian period begins (Garceau *et al.*, 1997). The roles of the multiple phosphorylations of FRQ are not known. However, phosphorylation of FRQ at Ser513 by an unidentified kinase affects protein stability and the length of the circadian period (Liu *et al.*, 2000).

A

N.c.	CK-1a	MTTMDLRVGNKYRIGRKIGSGSFGDIYLGNTNIIISGEEIAIKLESVKAKHPQLEYEARVYKSLAGGVGIPFV	71
S.c.	HRR25R.....H..L.....IRSR...D...S...RY.S.....I	68
H.s.	CK-1εE.....A..A.....C.T.....HI.SKF..MMQ.....SI	68
H.s.	CK-1δE.....D.AA.....C.T.....HI.SKI..MMQ.....TI	68
D.m.	DBTE.....L.....T.NT.....CIRT.....HI.SKF..TMQ.....RI	68
H.s.	CK-1α	MASSSSGSKAEFI..G..KLV.....AI..TN.....Q..R.....L..SKL..I..Q.....HI	77
N.c.	CK-1b	MASS..SSNV..VH..V.K...E...V.FE...LLNNQV...F.PR.SDA...RD.Y.T..I.V.CP...N	73
N.c.	CK-1a	RWFGTECDYNAMVLDLLGPSLEDLNFCNRKFSKLTVLLADQLISRIEYIHAKSFIHRDIKPDNFMGIGKRGNGV	148
S.c.	HRR25	..F.R.G...AM.ID.....Y.H.R..F..IM..L.MFC..Q...GR.....V.R..ST.	145
H.s.	CK-1ε	K.C.A.G...VM.ME.....F.S.K.....M.....S.N.....V.....L..K..L	145
H.s.	CK-1δ	..C.A.G...VM.ME.....F.S.S.....M.....S.N.....V.....L..K..L	145
D.m.	DBT	I.C.S.G...VM.ME.....F.S.R.....M...D...SRD.....L..K..L	145
H.s.	CK-1α	..Y.Q.K...VL.MD.....F.S.R.TM...M...M...V.T.N.....RHC.KL	151
N.c.	CK-1b	YY..Q.GLH..IL..ID.....DH...R...VMV.K.ML..VQT..E.NL.Y.....I.RQGTKTAN	150
N.c.	CK-1a	--NVIDFGLAKKYRDPKTHFHIPYRENKNLTGTARYASINTHLGVEQSRDDMESLGYVMLYFCRGSFPWQGLKAAT	221
S.c.	HRR25	--H.....S....FN..R.....S.....V.....L.....LI...K.....TT	218
H.s.	CK-1ε	--YI.....AR..Q.....V.....L.....LM..NL	218
H.s.	CK-1δ	--YI.....AR..Q.....V.....L.....LM..NL	218
D.m.	DBT	--YI.....ARSLK.....V.....L.....LM..NL.A.....N	218
H.s.	CK-1α	--FL.....NR.RQ.....V.A.....LM..N.T.....	224
N.c.	CK-1b	VIH.V...M..Q.....KQ.....R.S.S...M.....R.....L.A..H.FM..L..G.....	225
N.c.	CK-1a	ATKKQKYDRIMEKKMTTPTEVLCRGFPNEFAIYLNYSRLRFDDKPDYSYLRKIFRDLFVREGFQYDYVDFWTVYKY	298
S.c.	HRR25	TT.....LNVSVE...S.L.L..QE.MA.CKNLK..E...LF.ARL.KD.SIKLEYHN.HL...MLRY	295
H.s.	CK-1ε	..R...E..S...S..I...K.Y.S..ST..NFC.....QL..N..H.Q..S..YV...NML.F	295
H.s.	CK-1δ	..R...E..S...S..I...K.Y.S..ST..NFC.....QL..N..H.Q..S..YV...NML.F	295
D.m.	DBT	..N.R...E..S...LS.SIV..K...S..VN..NFC.QMH..QR..CH..L..N..H.L..T..YV...NLL.F	295
H.s.	CK-1α	..R...E..S...S.FV...K...A..M.N.C.G...EEA..M...QL..I...RTL.N.HQ.YT...TML.Q	301
N.c.	CK-1b	..N....EK.G...Q...AIKD..E...EQ..Q..T.V.N.G.E.Q...D...EL.SQALKDA.GVE.GEY...MKVKN	302

B



C

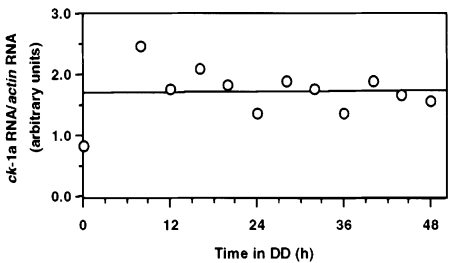


Fig. 1. Identification of *N.crassa* casein kinases 1, *N.c.* CK-1a and *N.c.* CK-1b. (A) Sequence alignment of amino acid residues 1–298 of *N.c.* CK-1a and residues 1–302 of *N.c.* CK-1b with the N-terminal kinase domains of selected members of the casein kinase 1 family. The amino acid sequence of *N.c.* CK-1a is shown in the upper lane. Residues identical to *N.c.* CK-1a are indicated by a dot. (B) Phylogenetic relationship of members of the casein kinases 1 family. *D.m.* DBT: *D.melanogaster* DOUBLETIME; *M.a.* TAU: *M.auratus* casein kinase 1 TAU; *S.c.* HRR25, *S.c.* CK 1, *S.c.* CK 2 and *S.c.* CK 3: *S.cerevisiae* casein kinases 1; *H.s.* CK1α, *H.s.* CK1δ and *H.s.* CK1ε: *Homo sapiens* casein kinases 1α, 1δ and 1ε. (C) *ck-1a* RNA levels are constant throughout a circadian day. Light-grown *Neurospora* cultures were transferred to constant darkness (DD) and samples were harvested after the indicated time periods. From each sample total RNA was isolated and cDNA was prepared by using random hexamers for priming. Relative levels of *ck-1* RNA and *actin* RNA were determined by reverse transcription and quantitative RT-PCR using the 5' nuclease assay detection system (TaqMan™) as described in Materials and methods. For calibration, cDNA was prepared from serial dilutions of RNA from light-grown tissue. The relative ratios of *ck-1/actin* were plotted versus the corresponding time in DD.

FRQ contains two PEST-like sequences, PEST-1 and PEST-2 (Morrow and Dunlap, 1994). PEST sequences are rich in proline (P), aspartic and glutamic acid (E), serine (S), threonine (T) (Rogers *et al.*, 1986). In many cases PEST sequences are implicated in the regulation of protein turnover (Rechsteiner and Rogers, 1996; Marchal *et al.*, 2000; Roth and Davis, 2000).

Here we show that PEST-1 and PEST-2 of FRQ are targets for phosphorylation *in vitro* by CK-1a and CK-1b, two newly identified casein kinases 1 of *N.crassa*. Deletion of PEST-1 results in loss of the circadian rhythm of conidiation. FRQΔPEST-1 is expressed at high levels; it is hypophosphorylated and significantly more stable than authentic FRQ. Unexpectedly, *frq*ΔPEST-1 RNA and FRQΔPEST-1 protein as well as WC-1 protein oscillate under constant conditions with a period of ~28 h. The deletion of the PEST-1 sequence in FRQ dissociates the

overt conidiation rhythm of *Neurospora* from molecular oscillations of critical clock components.

Results

Phosphorylation of PEST sequences in FRQ by casein kinases 1

The length of a circadian period in *Drosophila* is regulated via phosphorylation of the clock protein PER by DBT, a homolog of human casein kinase 1ε (CK-1ε) (Kloss *et al.*, 1998). Searching the *Neurospora* genome we found two CK-1 homologs, CK-1a, which is closely related to DBT, and CK-1b (Figure 1A and B). Attempts to inactivate CK-1a by repeat-induced point mutations (RIPs) (Selker and Garrett, 1988) were unsuccessful (data not shown), suggesting that the gene may be essential for cell viability. To characterize expression of CK-1a *Neurospora* was

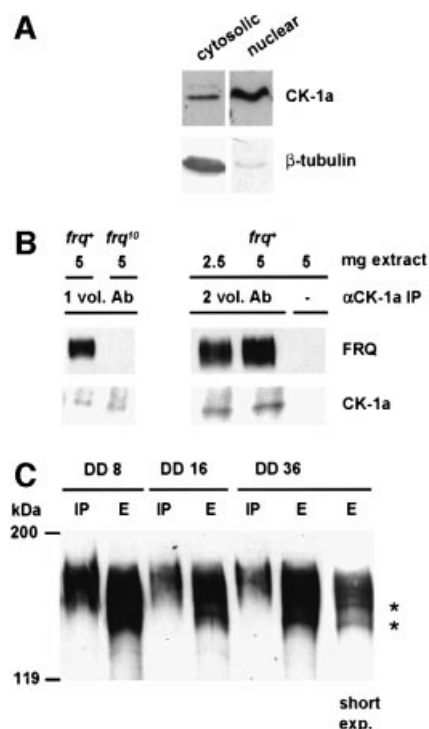


Fig. 2. CK-1a is in a complex with FRQ. (A) Subcellular localization of CK-1a. Light-grown *Neurospora* cells were fractionated as described (Luo *et al.*, 1998). Cytosolic and nuclear fractions (200 µg each) were analyzed by SDS-PAGE and western blotting with affinity-purified antibodies against CK-1a (αCK-1a) (upper panels) and monoclonal antibodies against *Neurospora* tubulin (lower panels). (B) Co-immunoprecipitation of FRQ with CK-1a. Total cell extracts from the indicated strains were subjected to immunoprecipitation with affinity-purified CK-1a antibodies. 1 vol. and 2 vol. Ab correspond to the amounts of αCK-1a purified from 0.2 ml and 0.4 ml serum, respectively. Samples (1/3 of total) were analyzed by SDS-PAGE and western blotting with the monoclonal antibody FRQm3G11 (upper panels) and αCK-1a (lower panels). (C) CK-1a is in a complex with hyperphosphorylated FRQ. Total cell extracts were prepared from cells that were grown for the indicated time periods in DD. Samples were subjected to immunoprecipitation with αCK-1a (2 vol. Ab). Total cell extracts (E) and immunoprecipitates (IP) were analyzed by SDS-PAGE and western blotting with FRQm3G11. Right lane: short exposure of the FRQ signal in DD36 extract. Low phosphorylated forms of FRQ are indicated by asterisks.

grown in the dark. Samples were harvested at 4 h intervals over a time course of 48 h and *ck-1a* RNA was analyzed by reverse transcription and real time quantitative polymerase chain reaction (RT-PCR) (Figure 1C) and northern blotting (not shown). *ck-1a* RNA was expressed at constant levels throughout the 48 h period.

CK-1a does not contain a canonical nuclear import signal. Yet, when *Neurospora* cells were fractionated CK-1a was found in the cytosolic and in the nuclear fraction, similar to DBT in *Drosophila* (Figure 2A). To investigate whether CK-1a interacts with FRQ *in vivo*, total protein extracts were prepared from light-grown *Neurospora* and subjected to immunoprecipitation with affinity-purified antibodies against CK-1a (Figure 2B). CK-1a antibodies coprecipitated specifically FRQ while WC-1 was not precipitated (not shown). *Neurospora* cells were transferred to darkness and samples were harvested 8, 16 and 36 h after the light-dark (LD) transfer. Total cell lysates were prepared and subjected to immunoprecipitation with

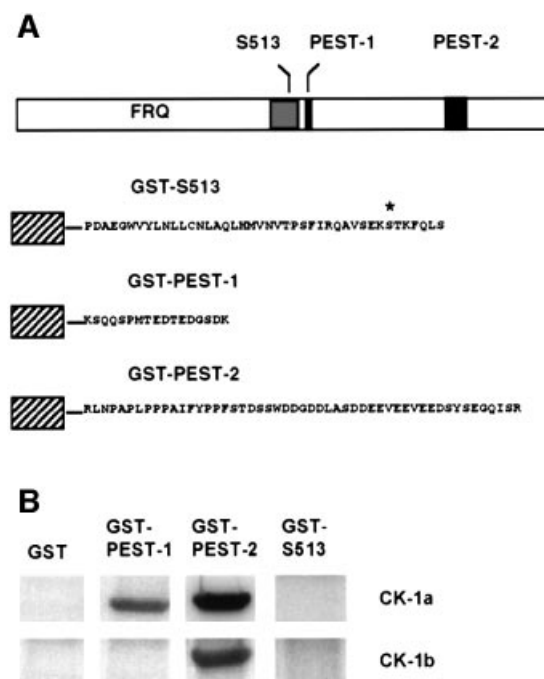


Fig. 3. Phosphorylation of PEST-like elements in FRQ by recombinant CK-1a and CK-1b. (A) Construction of GST fusion proteins. FRQ is schematically outlined in the upper portion. Two PEST-like elements were identified with 'PEST-find' (<http://www.at.emblnet.org/emblnet/tools/bio/PESTfind>). PEST-1 (score 16.4) and PEST-2 (score 11.3) are indicated with black boxes. The gray box corresponds to residues from 478–519 of FRQ. Ser513 is indicated by an asterisk. FRQ-derived sequences that were fused to GST (hatched box) are shown in the lower part. (B) Specific phosphorylation of GST-PEST-1 by CK-1a and of GST-PEST-2 by CK-1a and CK-1b. The indicated GST fusion proteins (500 ng) were incubated with 50 ng recombinant His-tagged CK-1a (upper panel) and CK-1b (lower panel) for 60 min at 37°C in the presence of [³²P]γ-ATP (2 Ci/mmol). Samples were analyzed by SDS-PAGE and autoradiography.

affinity-purified antibodies against CK-1a (Figure 2C). FRQ coimmunoprecipitated with CK-1a using extracts prepared at DD8, DD16 and DD36, suggesting that it was in a complex with FRQ throughout a circadian day. However, separation of the immunoprecipitates on long SDS gels revealed that forms of FRQ with low electrophoretic mobility were preferentially coprecipitated with CK-1a, suggesting that CK-1a is in a complex with highly phosphorylated FRQ. Thus, CK-1a either associates specifically with previously phosphorylated forms of FRQ or binds and rapidly phosphorylates FRQ such that complexes between CK-1a and non-phosphorylated FRQ may not accumulate.

We cloned the cDNAs encoding CK-1a and CK-1b, expressed His-tagged versions of both kinases in *Escherichia coli* and purified the recombinant proteins by Ni-NTA chromatography (see Materials and methods). The recombinant casein kinases were active in auto-phosphorylation assays and efficiently phosphorylated β-casein (not shown).

In *Saccharomyces cerevisiae*, casein kinases 1 phosphorylate uracil permease within a PEST sequence and, thereby trigger turnover of the protein (Marchal *et al.*, 2000). FRQ contains two PEST-like elements, PEST-1 and PEST-2 (Figure 3A, upper portion), which are conserved in FRQ proteins from other fungi (Morrow and Dunlap, 1994). The PEST-find scores of 16.4 and 11.3,

respectively, are significantly above the threshold level (5.0) (Rogers *et al.*, 1986). PEST-1 is located in the central portion of FRQ and comprises a short confined segment of 17 amino acid residues. PEST-2 is located in the C-terminal portion and is 50 amino acid residues in length. We investigated whether the PEST sequences of FRQ are targets for phosphorylation by CK-1a and CK-1b. Fusion proteins between glutathione *S*-transferase (GST) and PEST sequences were constructed, expressed and purified. GST-PEST-1 and GST-PEST-2 contain the predicted PEST-like sequences comprising residues 544–560 and 843–893 of FRQ, respectively (Figure 3A, lower portion). Neither CK-1a nor CK-1b phosphorylated GST, which was used as a control (Figure 3B). Similarly, purified maltose-binding protein was not phosphorylated by the recombinant casein kinases (data not shown). GST-PEST-1 was specifically phosphorylated by CK-1a but not by CK-1b (Figure 3B). GST-PEST-2 was efficiently phosphorylated by CK-1a and with lower efficiency by CK-1b. Thus, PEST-2 appears to be a target for both casein kinases 1, while PEST-1 is a candidate sequence for specific phosphorylation of FRQ by CK-1a, the *Neurospora* homolog of *Drosophila* DBT.

FRQ is phosphorylated at Thr501, Ser513 and Ser519 (Liu *et al.*, 2000). To test whether these sites are phosphorylated by the *Neurospora* casein kinases 1, we constructed GST-S513, a fusion protein containing residues 478–519 of FRQ. Recombinant GST-S513 was neither phosphorylated by CK-1a nor by CK-1b (Figure 3B). Based on the evidence from *in vitro* phosphorylation assays, which must be interpreted with caution, FRQ might be phosphorylated by multiple kinases. However, a bona fide role of different kinases in the circadian system of *Neurospora* remains to be established.

Deletion of PEST-1 in FRQ results in loss of overt rhythmicity

Since PEST-1 was specifically phosphorylated by CK-1a, we deleted the region encoding PEST-1 (17 amino acid residues) from *frq* and expressed the mutant gene under control of its own promoter in a *frq*-less *Neurospora* strain (*frq*¹⁰) (Aronson *et al.*, 1994). When the resulting strain, *frq*ΔPEST-1, was grown on race tubes in DD, no rhythmic conidiation (banding) was observed (Figure 4A). However, *frq*ΔPEST-1 was growing with unregulated conidiation, while *frq*¹⁰ did not form conidia under these conditions. A control strain transformed with the wild-type *frq* gene showed a normal banding pattern with a periodicity of ~22 h (not shown). Thus, deletion of the PEST-1 region of FRQ results in the loss of overt rhythmicity.

Why is conidiation of *frq*ΔPEST-1 arrhythmic in DD? To assess whether *frq*ΔPEST-1 had generally lost the ability to form conidial bands, race tubes were inoculated with *frq*⁺, *frq*ΔPEST-1 and *frq*¹⁰ and then exposed to symmetric LD cycles of different length (Figure 4B). *frq*⁺ showed banding in LD cycles while *frq*¹⁰ does not band under these conditions (Figure 4B, bottom panel and data not shown), confirming that light-regulated conidial banding requires functional FRQ (Chang and Nakashima, 1997; Merrow *et al.*, 1999; Lakin-Thomas and Brody, 2000). Interestingly, *frq*ΔPEST-1 banded in

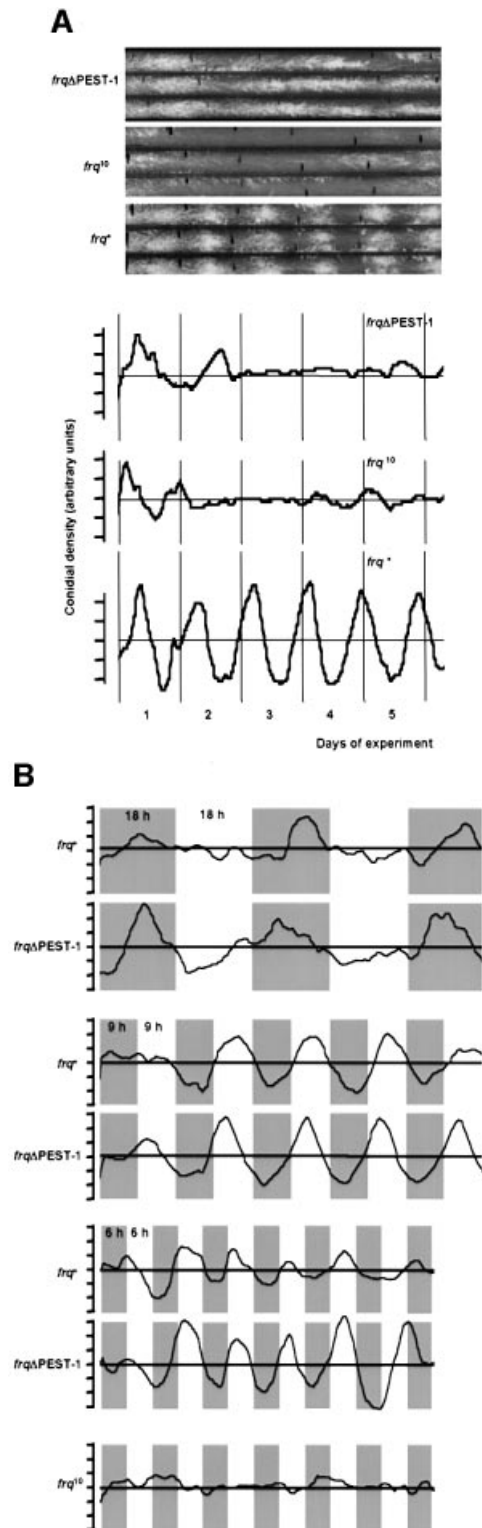


Fig. 4. *frq*ΔPEST-1 has lost the overt conidiation rhythm. (A) Race tubes were inoculated with *frq*ΔPEST-1, wild type (*frq*⁺) and *frq* null (*frq*¹⁰) and incubated at 25°C in DD. Upper panel: three replicate race tubes from each strain are shown. Lower panel: average conidial densities of triplicate race tubes. (B) *frq*⁺ and *frq*ΔPEST-1 show driven conidial banding in 18 h/18 h, 9 h/9 h and 6 h/6 h LD cycles. Race tubes inoculated with the indicated strains were exposed to symmetrical LD cycles. The conidial densities of race tubes were determined and the averages of triplicates were plotted versus the time. The durations of the LD cycles are indicated. The gray background bars correspond to dark periods. Data of *frq*¹⁰ are shown only from the 6 h/6 h LD cycle; *frq*¹⁰ displayed no conidiation rhythm under all tested conditions.

LD cycles that varied in length between 18 h/18 h and 6 h/6 h. In LD cycles conidiation is driven by light. In 12 h/12 h and longer cycles conidial bands are formed towards the end of the dark phase, while in shorter cycles conidiation, though initiated in the dark, is pushed into the light phase of the following day. This indicates that *frq*ΔPEST-1 has the capacity to form bands when conidiation is driven by light. However, since *frq*ΔPEST-1 does not band in DD it has apparently lost the ability to coordinate conidiation in a temporal fashion under constant conditions.

Physical and functional properties of *FRQ*ΔPEST-1

To characterize expression of the mutant *FRQ*ΔPEST-1 protein, total cell extracts were analyzed from wild-type and mutant strains that were grown in constant light (LL) (Figure 5A, left panel). As estimated by densitometry *FRQ*ΔPEST-1 was expressed at ~3-fold higher levels than *FRQ* (not shown). Levels of *frq*ΔPEST-1 RNA were slightly lower than levels of *frq*⁺ RNA (Figure 5A, right panel), suggesting that the elevated levels of *FRQ*ΔPEST-1 were due to an increased stability of the mutant protein (see below).

The phosphorylation pattern of *FRQ*ΔPEST-1 differed, however, significantly from that of *FRQ*. Light-grown control cells accumulated three major forms of *FRQ* that differed in electrophoretic mobility (Figure 5B). In contrast, only two major forms of *FRQ*ΔPEST-1 were observed. The mobility shifts were due to phosphorylation, since treatment of *FRQ* with phosphatase prior to SDS-PAGE resulted predominantly in a form of *FRQ* that co-migrated with *in vitro* synthesized, non-phosphorylated *FRQ*. Similarly, phosphatase treatment of *FRQ*ΔPEST-1 resulted in a species that co-migrated with *in vitro* synthesized *FRQ*ΔPEST-1 (Figure 5B). Due to the deletion of the PEST-1 region non-phosphorylated *FRQ*ΔPEST-1 migrated with a slightly higher mobility than non-phosphorylated *FRQ*.

Thus, the absence of one of the three phosphorylated species in *FRQ*ΔPEST-1 indicates that the PEST-1 region is either a direct target for phosphorylation and/or required to direct phosphorylation at other sites.

We then analyzed physical properties of *FRQ*ΔPEST-1. Deletion of PEST-1 did not alter the subcellular localization of the mutant protein as determined by subcellular fractionation and western blotting (not shown). Sedimentation analysis in sucrose density gradient suggested that the oligomeric state of *FRQ* (Denault *et al.*, 2001) was not affected by deletion of the PEST-1 sequence (not shown).

When *Neurospora* is transferred from dark to light, synthesis of *frq* and *wc-1* RNA are induced (Crosthwaite *et al.*, 1997; Linden and Macino, 1997). The levels of light-induced RNAs are dependent on *FRQ* (Heintzen *et al.*, 2001; Merrow *et al.*, 2001). To assess functional properties of *FRQ*ΔPEST-1, we measured light inducibility of *frq* RNA and *wc-1* RNA. In *frq*⁺ cells both RNAs were rapidly induced by light (Figure 6A, left panels). As expected, RNA levels reached a maximum after ~10 min and adapted to low levels after longer light exposure of the cells (Arpaia *et al.*, 1999; Heintzen *et al.*, 2001). Similarly, *frq*ΔPEST-1 RNA and *wc-1* RNA were rapidly induced by light in *frq*ΔPEST-1 cells and adapted subsequently to lower levels (Figure 6A, right panels).

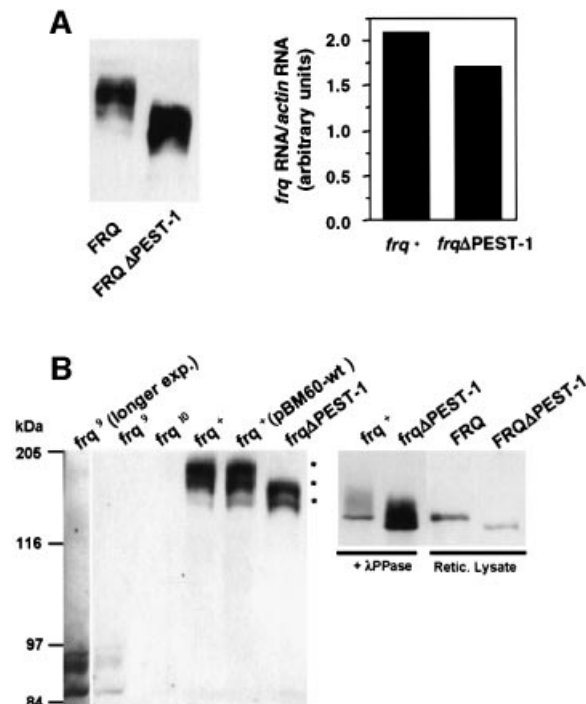


Fig. 5. Expression and phosphorylation profile of *FRQ*ΔPEST-1. (A) Left panel: protein extracts (200 µg) from light-grown *frq*⁺ and *frq*ΔPEST-1 were analyzed by SDS-PAGE and western blotting with the monoclonal anti-*FRQ*m3G11 antibody (α*FRQ*) (Merrow *et al.*, 2001). Right panel: total RNA was prepared from light-grown strains. *frq*⁺ and *frq*ΔPEST-1 RNA were quantified by RT-PCR (as described in the legend to Figure 1C). (B) *FRQ*ΔPEST-1 is hypophosphorylated. Left panel: total protein extracts from the indicated light-grown strains. Major forms of *FRQ* that differ in electrophoretic mobility are indicated by asterisks. *frq*⁹: frameshift mutant with truncated *FRQ*; *frq*¹⁰: *frq* null mutant; *frq*⁺: wild-type *frq*; *frq*⁺ (pBM60-wt): *frq*¹⁰ transformed with pBM60 harboring wild-type *frq*; *frq*ΔPEST-1: *frq*¹⁰ transformed with pBM60 harboring *frq*ΔPEST-1. Central panel: protein extracts were subjected to immunoprecipitation with α*FRQ* and the precipitates were treated with 1000 U of λ-phosphatase (λ-PPase). Right panel: *FRQ* and *FRQ*ΔPEST-1 were synthesized by coupled *in vitro* transcription/translation in reticulocyte lysate (Retic. lysate). Samples were analyzed by SDS-PAGE and immunoblotting with α*FRQ*.

Thus, *FRQ*ΔPEST-1 supports inducibility by light as well as adaptation to light of *wc-1* and *frq*ΔPEST-1 RNA.

FRQ facilitates expression of *WC-1* in a post-transcriptional manner (Lee *et al.*, 2000; Cheng *et al.*, 2001). Since *FRQ*ΔPEST-1 was expressed at high levels, we compared expression levels of *WC-1* in light-grown *frq*ΔPEST-1 and *frq*⁺. Equal amounts of total cell extracts were analyzed by SDS-PAGE and western blotting (Figure 6B). *WC-1* was expressed in *frq*ΔPEST-1 at significantly higher levels than in *frq*⁺. *wc-1* RNA levels were similar in both strains (not shown), indicating that *FRQ*ΔPEST-1 supports the expression of *WC-1* in a manner similar to that proposed for *FRQ* (Lee *et al.*, 2000).

Synthesis of *frq* RNA is repressed when *Neurospora* cells are transferred from light to darkness. We measured the kinetics of RNA repression in *frq*⁺ and *frq*ΔPEST-1 (Figure 6C). In both strains *frq* RNA levels decreased within 30 min after LD transfer. When *frq*⁹ was transferred from light to dark, no significant reduction in the amount of *frq*⁹ RNA was observed (not shown and Merrow *et al.*, 1999). This demonstrates that deletion of PEST-1 does not

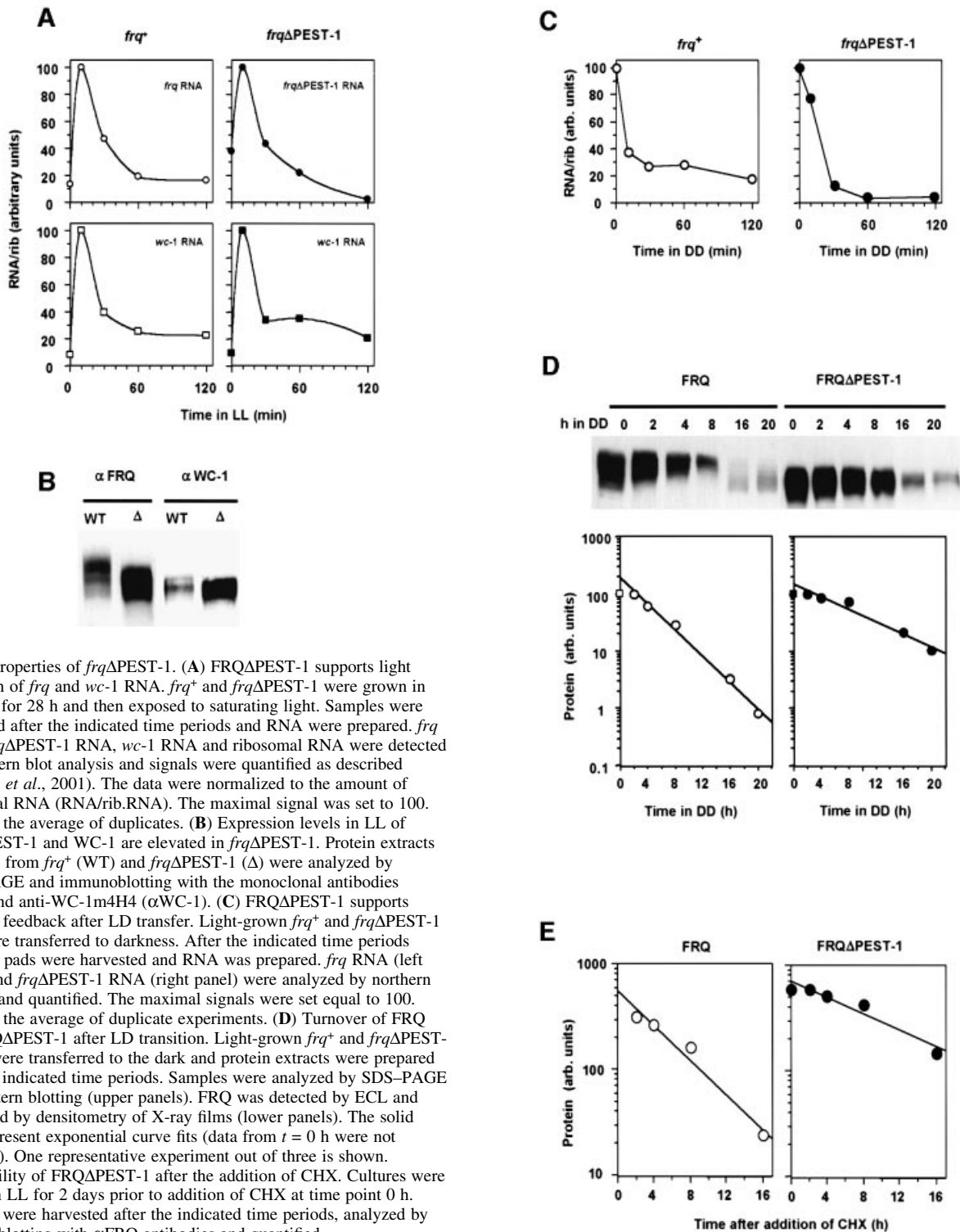


Fig. 6. Properties of *frqΔPEST-1*. (A) FRQΔPEST-1 supports light induction of *frq* and *wc-1* RNA. *frq*⁺ and *frqΔPEST-1* were grown in the dark for 28 h and then exposed to saturating light. Samples were harvested after the indicated time periods and RNA were prepared. *frq* RNA, *frqΔPEST-1* RNA, *wc-1* RNA and ribosomal RNA were detected by northern blot analysis and signals were quantified as described (Morrow *et al.*, 2001). The data were normalized to the amount of ribosomal RNA (RNA/rib.RNA). The maximal signal was set to 100. Data are the average of duplicates. (B) Expression levels in LL of FRQΔPEST-1 and WC-1 are elevated in *frqΔPEST-1*. Protein extracts (200 μg) from *frq*⁺ (WT) and *frqΔPEST-1* (Δ) were analyzed by SDS-PAGE and immunoblotting with the monoclonal antibodies αFRQ and anti-WC-1m4H4 (αWC-1). (C) FRQΔPEST-1 supports negative feedback after LD transfer. Light-grown *frq*⁺ and *frqΔPEST-1* cells were transferred to darkness. After the indicated time periods mycelial pads were harvested and RNA was prepared. *frq* RNA (left panel) and *frqΔPEST-1* RNA (right panel) were analyzed by northern blotting and quantified. The maximal signals were set equal to 100. Data are the average of duplicate experiments. (D) Turnover of FRQ and FRQΔPEST-1 after LD transition. Light-grown *frq*⁺ and *frqΔPEST-1* cells were transferred to the dark and protein extracts were prepared after the indicated time periods. Samples were analyzed by SDS-PAGE and western blotting (upper panels). FRQ was detected by ECL and quantified by densitometry of X-ray films (lower panels). The solid lines represent exponential curve fits (data from *t* = 0 h were not included). One representative experiment out of three is shown. (E) Stability of FRQΔPEST-1 after the addition of CHX. Cultures were grown in LL for 2 days prior to addition of CHX at time point 0 h. Samples were harvested after the indicated time periods, analyzed by western blotting with αFRQ antibodies and quantified.

compromise the ability of the cells to negatively feed back on *frq* transcription.

We then measured the kinetics of turnover of FRQ and FRQΔPEST-1 protein. Light-grown cells were transferred to dark to repress new synthesis of *frq* and *frqΔPEST-1* RNA. Subsequently samples were analyzed over a time course of 20 h. Following the LD transfer FRQ and FRQΔPEST-1 were hyperphosphorylated. Due to changes in the phosphorylation pattern it was difficult to compare

protein levels between 0 and 2 h (Figure 6D, upper panel). Yet, levels did not appear to change significantly. Subsequently FRQ was rapidly degraded with a half-life of 2.7 ± 0.2 h (Figure 6D, lower panels). In comparison, degradation of FRQΔPEST-1 was slow. The half-life for turnover was 5.6 ± 0.3 h, indicating that the mutant protein was significantly more stable than authentic FRQ. After 16 h in DD hyperphosphorylated FRQ was completely degraded and new synthesis of low phos-

phorylated forms of FRQ was observed, demonstrating that a new circadian oscillation had commenced. No new synthesis of FRQΔPEST-1 occurred in the observed 20 h time period.

To confirm the differences in protein stability, *frq* and *frq*ΔPEST-1 cells were treated with cycloheximide (CHX) to inhibit protein synthesis, and samples were analyzed over a 16 h period. Again, turnover of FRQ was faster than the turnover of FRQΔPEST-1 (Figure 6E). The overall kinetics of degradation of both proteins were, however, slower in the presence of CHX than in the absence of the drug. FRQ was degraded with a half-life of 3.7 h, while the half-life of degradation of FRQΔPEST-1 was 7.7 h. The slower degradation kinetics may be due to pleiotropic effects of CHX and indicate that ongoing protein synthesis is required for the turnover of FRQ at physiological rates.

In summary, the data show that deletion of PEST-1 results in a pronounced stabilization of FRQΔPEST-1 protein.

Rhythmicity of *frq*ΔPEST-1 on the molecular level

The deletion of PEST-1 does not interfere with crucial FRQ-dependent properties such as regulation of conidiation by light, adaptation of RNA expression levels to light and negative feedback on *frq* RNA synthesis after LD transfer of cells (see above). We therefore tested whether components of the circadian clock are rhythmically expressed in *frq*ΔPEST-1 cells despite the lack of an overt conidiation rhythm. *frq*ΔPEST-1 cells were grown in liquid culture and harvested at 3 h intervals spanning a time period of 48 h. As a control, *frq*⁺ cells were harvested at 4 h intervals over the same period. Total cell lysates of each time point were subjected to western blot analysis. As expected, FRQ was hyperphosphorylated and rapidly turned over (Figure 7A and B, upper panels). New synthesis of unphosphorylated FRQ was observed after 16 h in DD. Surprisingly FRQΔPEST-1 was also rhythmically expressed (Figure 7A and B, central panels). After LD transfer FRQΔPEST-1 protein showed the characteristic mobility shifts due to progressive phosphorylation. The phosphorylated protein was slowly degraded over an extended time period. After 24 h in DD low phosphorylated FRQΔPEST-1 protein was newly synthesized. This indicates that the negative feedback was relieved and a new circadian cycle had started. However, compared to synthesis of FRQ in control cells, the phase of FRQΔPEST-1 was delayed by ~8 h. This is presumably due to the high expression levels of FRQΔPEST-1 in LL and its increased stability. The newly synthesized FRQΔPEST-1 was then gradually phosphorylated and slowly degraded. It was still detectable after 48 h, indicating that the circadian period of *frq*ΔPEST-1 was longer than 24 h.

WC-1 was also expressed in a rhythmic fashion in *frq*ΔPEST-1 cells (Figure 7A and B, lower panels). WC-1 reached a first maximum 6 h after LD transfer and a second maximum after 33 h in DD. WC-1 levels were minimal at DD21 and DD48. This suggests that the circadian clock was oscillating with a period of ~27 h in *frq*ΔPEST-1 cells.

To analyze circadian rhythmicity on the transcriptional level, RNA from *frq*⁺ and *frq*ΔPEST-1 cells was prepared at 4 h intervals over a time course of 60 h. *frq*⁺ and *frq*ΔPEST-1 RNA was quantified by RT-PCR (Figure 8A,

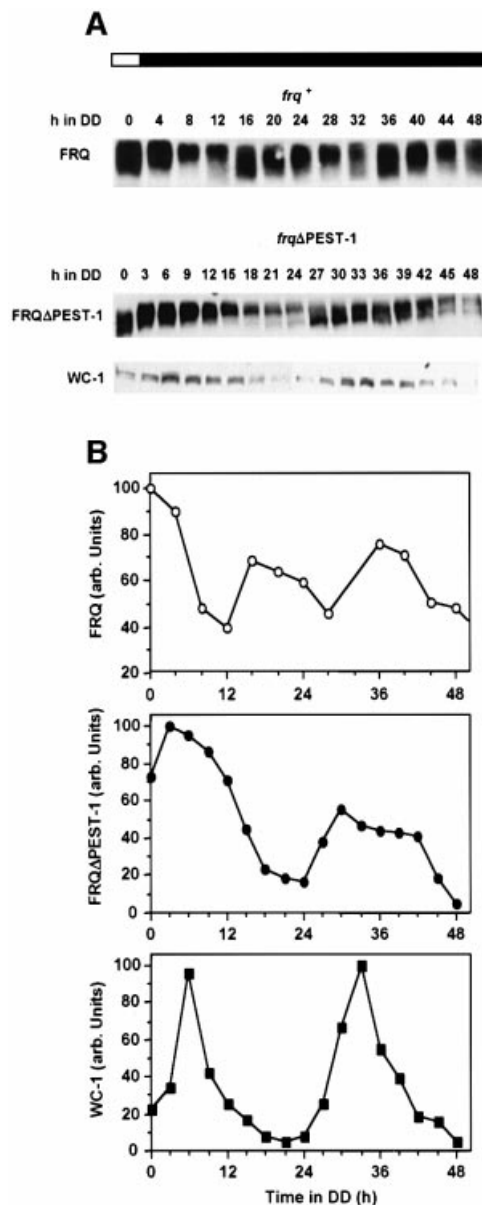


Fig. 7. Circadian oscillation of FRQΔPEST-1 and WC-1 in *frq*ΔPEST-1. (A) Light-grown *frq*⁺ and *frq*ΔPEST-1 were transferred to darkness (black bar). Samples were harvested at 4 h intervals (*frq*⁺) and 3 h intervals (*frq*ΔPEST-1) over a time course of 48 h. Protein extracts were prepared and analyzed by SDS-PAGE, western blotting and ECL. FRQ (upper panel) and FRQΔPEST-1 (central panel) were detected with αFRQ antibodies and WC-1 was detected with αWC-1 antibodies (lower panel). (B) The indicated antigens were quantified by densitometry of ECL signals on selected X-ray films. The maximal signals were set to 100.

upper panel). *ck-1a* RNA, which is not rhythmically expressed (see above), was also quantified and data were used for normalization. Within the observed time course *frq*⁺ RNA levels peaked three times, around DD12, DD32 and DD56, in agreement with a circadian period of 22 h. *frq*ΔPEST-1 RNA was also rhythmically expressed (Figure 8A, lower panel and panel B). However, *frq*ΔPEST-1 RNA peaked only twice, at DD28 and DD56. This indicates that the circadian period was lengthened to ~28 h. The appearance of the first peak of

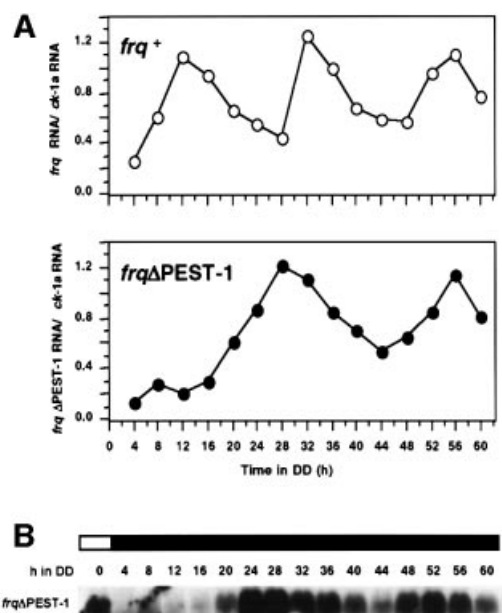


Fig. 8. Rhythmic expression of *frq*ΔPEST-1 RNA. Light-grown *frq*⁺ and *frq*ΔPEST-1 were transferred to the dark. Samples were harvested at 4 h intervals over a time course of 60 h and total RNA was prepared. (A) *frq*, *frq*ΔPEST-1 and *ck-1a* RNA was quantified by RT-PCR using the 5' nuclease assay detection system (TaqManTM). For calibration, cDNA was prepared from serial dilutions of RNA from light-grown tissue. The relative ratios of *frq*/*ck-1* and *frq*ΔPEST-1/*ck-1* were plotted versus the corresponding time in DD. (B) *frq*ΔPEST-1 RNA was analyzed by northern blotting.

*frq*ΔPEST-1 RNA at DD28 indicates that the phase was substantially delayed following the light-to-dark transfer.

Discussion

We show that CK-1a, a *Neurospora* homolog of *Drosophila* DBT interacts with FRQ *in vivo* and that CK-1a phosphorylates two PEST regions of FRQ *in vitro*. Deletion of the PEST-1 region in FRQ results in an arrhythmic conidiation phenotype. Yet, on the molecular level, *frq* RNA, as well as FRQ and WC-1 protein, oscillate with a circadian period of ~28 h. What causes the loss of overt rhythmicity and the lengthening of the period in *frq*ΔPEST-1? FRQ-dependent functions, such as light-dependent conidiation, repression of *frq* RNA synthesis upon LD transfer and adaptation of light-induced *frq* and *wc-1* RNA levels, are functional in *frq*ΔPEST-1. As with *frq*⁺, the lag between *frq*ΔPEST-1 RNA transcription and FRQΔPEST-1 protein synthesis comprises ~4 h and thus does not account for the observed period lengthening in *frq*ΔPEST-1. However, FRQΔPEST-1 is significantly more stable than FRQ and the mutant protein accumulates at high levels in light-grown cells. The circadian expression profile of FRQΔPEST-1 is broadened in a manner that corresponds to the observed period length in *frq*ΔPEST-1. The persistence of high levels of stable FRQΔPEST-1 leads apparently to a prolonged negative feedback. This may cause the pronounced phase delay in *frq*ΔPEST-1 RNA transcription and the lengthening of the circadian period.

Recently it was shown that the FRQ feedback loop is interconnected with WC-1 expression (Lee *et al.*, 2000).

Although the molecular mechanism is not yet fully understood, expression and rhythmicity of WC-1 are controlled by FRQ in a post-transcriptional manner (Lee *et al.*, 2000; Cheng *et al.*, 2001). WC-1 on the other hand is required for adequate expression levels of *frq* RNA (Crosthwaite *et al.*, 1997; Cheng *et al.*, 2001). The interconnected loops contribute to the robustness of the circadian oscillation (Cheng *et al.*, 2001). WC-1 levels are elevated in light-grown *frq*ΔPEST-1 cells and circadian oscillation of WC-1 is supported by FRQΔPEST-1 in DD. This suggests that the interconnected feedback loop is functional in *frq*ΔPEST-1.

It is surprising that *frq*ΔPEST-1 does not form conidial bands on race tubes although *frq*ΔPEST-1 RNA and FRQΔPEST-1 protein oscillate in DD. Thus, the endogenous molecular rhythm of *frq*ΔPEST-1/FRQΔPEST-1 is not accompanied by an overt conidiation rhythm, a phenomenon hitherto not described. The length of the circadian period of *frq*ΔPEST-1 inferred from the molecular oscillations (28 h) is similar to that of the long period mutant *frq*⁷ (29 h) (Feldman and Hoyle, 1973). Yet, *frq*⁷ bands on race tubes in DD. We have no explanation of why *frq*ΔPEST-1 has lost the ability to band in DD. However, the circadian expression profiles of clock proteins in *frq*ΔPEST-1 differ significantly from those in *frq*⁺ and *frq*⁷. Circadian expression profiles of FRQ and WC-1 are separated in *frq*⁺ and in *frq*⁷ (Lee *et al.*, 2000). In contrast, the temporal expression profile of FRQΔPEST-1 is broad due to its increased stability and it overlaps temporally with the peak of WC-1 expression. FRQΔPEST-1 levels reach a maximum at DD30 and remain high for ~12 h. Thus, it resembles for most of a circadian day the expression of FRQ in light-grown *frq*⁺ cells, which could explain why conidial banding is disturbed in *frq*ΔPEST-1.

Although *frq*ΔPEST-1 does not display an overt conidiation rhythm in DD, it forms conidial bands in LD cycles. In this respect, the strain resembles *frq*¹⁰ *qa-2p*-FRQ, where FRQ is constantly expressed at high levels (Merrow *et al.*, 2001). *frq*¹⁰ *qa-2p*-FRQ is arrhythmic in DD but bands in LD cycles. Rhythmic expression of WC-1 and of light-induced genes should be driven in LD cycles and may allow banding of strains which maintain high levels of FRQ throughout a circadian day.

What is the function of the PEST-1 element of FRQ? During the course of a circadian period FRQ is progressively phosphorylated and then degraded (Garceau *et al.*, 1997). Under continuous illumination three major forms of FRQ accumulate to a steady state, which correspond to a species that differ in their extent of phosphorylation. One of these forms is absent when the PEST-1 segment is deleted, suggesting that PEST-1 is a target for phosphorylation *in vivo*. FRQΔPEST-1 is significantly more stable than FRQ, demonstrating that PEST-1 affects protein turnover. No other function of FRQ is apparently affected by the deletion of PEST-1. The PEST-1 sequence therefore appears to be a functional element that regulates turnover of FRQ in response to phosphorylation. *Neurospora* CK-1a *in vitro* phosphorylates PEST-1. PEST sequences are often targets for phosphorylation and implicated in regulated protein turnover. In *S.cerevisiae*, uracil permease is phosphorylated within a PEST-like sequence by casein kinases 1, triggering

ubiquitylation, endocytosis and degradation of the plasma membrane transporter (Marchal *et al.*, 2000). Casein kinases phosphorylate serine and threonine residues in the environment of aspartic and glutamic acids residues (Flotow and Roach, 1991; Pulgar *et al.*, 1999). Since these residues are enriched in PEST sequences, they may in many cases be targets for phosphorylation by casein kinases.

Phosphorylation-dependent shifts in electrophoretic mobility of FRQ suggest that the protein is phosphorylated at multiple sites. FRQ contains a second PEST-like sequence that is phosphorylated by CK-1a and CK-1b *in vitro*. Whether the PEST-2 sequence has a function in protein turnover is currently under investigation. Recently Liu *et al.* (2000) reported phosphorylation of FRQ at three sites outside of the PEST sequences, Thr501, Ser513 and Ser519. *Neurospora* Ca/CaM-kinases were suggested to be involved in these phosphorylations (Yang *et al.*, 2001). These sites were not phosphorylated by CK-1a or CK-1b *in vitro* and the sequence contexts have no similarity to casein kinase 1 consensus sites. This raises the question whether FRQ is phosphorylated by more than one kinase *in vivo*. An exchange of Ser513 affected the stability of FRQ and the length of the circadian period in a similar manner as deletion of PEST-1 (Liu *et al.*, 2000). Whether the different phosphorylations of FRQ directly affect the kinetics of protein turnover is not known. Various steps in the lifetime of FRQ might be regulated by phosphorylation. If one of these steps is slowed down, this may indirectly result in slower kinetics of FRQ turnover.

Interconnected feedback loops are a key feature of circadian systems in all species (Loros and Dunlap, 2001; Reppert and Weaver, 2001; Williams and Sehgal, 2001). Casein kinases 1 might be conserved components, which are recruited by the circadian clocks to regulate period length via phosphorylation of components of the negative feedback loops.

Materials and methods

Strains, media and growth conditions

Neurospora standard laboratory strains used in this study were *frq*⁺ (*frq* wild type), *frq*⁷ (long period mutant strain with point mutation in FRQ), *frq*⁹ (frameshift mutant with truncated FRQ) and *frq*¹⁰ (*frq* null mutant). All strains carry the *bd* mutation (Loros *et al.*, 1986; Aronson *et al.*, 1994; Merrow *et al.*, 1999). The *Δwc-1;bd* strain was generated by RIP (Talora *et al.*, 1999), and crossed for *bd*, as previously described (Merrow *et al.*, 2001). Neither *wc-1* RNA nor WC-1 protein is detectable in this strain. For transformations the *bd;frq*¹⁰*;his-3* strain was used (Bell-Pedersen *et al.*, 1996).

Race tube medium contained 0.3% glucose, 1× Vogel's medium (Vogel, 1964), 0.5% L-arginine, 10 ng/ml biotin and 2% agar. For all other experiments, the standard medium was 2% glucose, 1× Vogel's, 0.5% L-arginine and 10 ng/ml biotin. For inoculation 8- to 10-day-old conidia from solid standard medium were harvested and for each circadian time point 25 ml of media in 100 ml flasks were inoculated with 3.5×10^6 conidia.

All liquid cultures were incubated in LL at 30°C for 1–2 days prior to transfer to DD at 25°C. Light induction was performed after cultivation of cells for 28 h in DD. For circadian time courses the cultures were pre-incubated with shaking in LL until a staggered light-to-dark transfer of the cultures was performed. Age-matched cultures were harvested by filtration and immediately frozen in liquid nitrogen.

Race tube analyses and circadian time courses

Race tubes were inoculated, germinated in LL for ~24 h and transferred either to DD or different light–dark cycles (LD) at 25°C. The fluence rate

was 20 μE delivered by cool white fluorescent bulbs. Race tubes were scanned and the conidial densities were quantified using the Chrono program (Roenneberg and Taylor, 2000). Circadian time courses were performed essentially as described (Aronson *et al.*, 1994).

Plasmid constructs and *Neurospora* transformations

Cloning and expression of CK-1a and CK-1b. The open reading frame (ORF) of CK-1a was amplified from *Neurospora* cDNA using the oligo nucleotide primer pair: CCCCCTCGACAAGGTACCATGACTACCATGGATCTC and CCCCAAGCTTGGCGCCGCTTATCGCAAAATCCTGTC. A *Bam*HI–*Hind*III fragment was cloned into pQE30. CK-1b was amplified with the primers CCCCCTCGACATGGCTTCC-TCGACGTCCTCCAAC and CCCCAAGCTTCAGCTGCAGCACAGGG and a *Sall*–*Hind*III fragment was cloned into pQE30. The cloned cDNAs were sequenced and His-tagged versions of the recombinant kinases were expressed in *E. coli* and purified by Ni-NTA chromatography.

Construction of GST-fusion proteins: fragments encoding residues 478–519 (S513), 544–560 (PEST-1) and 843–893 (PEST-2) of FRQ were amplified by PCR. A *Bam*HI site was introduced in frame at the 5' end and a stop codon and *Not*I site at the 3' end. The fragments were cloned into pGEX4T2.

The ORF of the large form of FRQ was cloned into pGEM4 resulting in pGEM4-ORF2. In pGEM4ORF2ΔPEST-1 the region encoding PEST-1 (1630–1680) was replaced with TGGCGCCGC.

pBM60-wt contains the *Clal*-fragment (8.67 kbp) that is necessary for circadian rhythmicity (McClung *et al.*, 1989). An exchange of the *Afl*III–*Sfi*I fragment with the deleted PEST-1 region resulted in pBM60/*frq*ΔPEST-1. Spheroplasts of *frq*¹⁰*;bd;his-3* were generated as described, targeted transformations at the *his-3* locus were performed with pBM60/*frq*ΔPEST1 and pBM60-wt (wild-type control) as described (Madi *et al.*, 1994). Several mutant clones were selected and microconidia were generated for homogenous strains.

RNA analysis

RNA was prepared essentially as described except that only a single phenol extraction was performed (Crosthwaite *et al.*, 1995). RNA was run on 1.2% agarose formaldehyde gels and blotted onto Nylon membranes (HybondN, Amersham Corp.) (Merrow *et al.*, 1997). Riboprobes were used to probe the blots for *frq*, *wc-1* and ribosomal RNA as described (Crosthwaite *et al.*, 1995; Merrow *et al.*, 2001).

Reverse transcription and quantitative PCR (quantitative RT-PCR): cDNA was synthesized from 2 μg total RNA using random hexamers and treated with DNase. Duplicate reactions (50 μl) containing cDNA equivalent to 0.1 μg RNA were performed in 96-well plates with the GeneAmp® 5700 Sequence Detection System (Applied Biosystems) using the following primer pairs (900 nM) and TaqMan probes (250 nM):

Frequency: 5'-TTGTAATGAAGGTGTCCGAAGGT-3' (*frq* forw-primer)

5'-GGAGGAAGAAGCGGAAAACG-3' (*frq* rev-primer)

5'-FAM—ACCTCCAATCTCCGAACCTGCCTG-3' (*frq* TaqMan)

Casein kinase 1a:

5'-TTACTGGCACTGCCCGTTATG-3' (ck-1 forw-primer)

5'-CGTAGCCAAGGGACTCCATGT-3' (ck-1 rev-primer)

5'-FAM—CTCCATCAACACGATCTCGGCG-3' (ck-1 TaqMan)

Actin:

5'-AATGGGTCGGGTATGTGCAA-3' (actin forw-primer)

5'-CTTCTGGCCCATACCGATCAT-3' (actin rev-primer)

5'-FAM—CAGAGCTGTTTTCCCTTCCATCGTTGGT-3' (actin TaqMan)

RNA from light-grown cells was stepwise diluted (2, 0.2, 0.02, 0.002, 0.0002 μg) and used to generate quantitative RT-PCR calibration curves for each primer system.

Protein analyses

Protein was extracted from mycelia by previously described methods (Garceau *et al.*, 1997). Total protein (300 μg) per lane was subjected to SDS-PAGE on 7.5% gels for FRQ and WC-1, and 16% gels for CK-1a.

Western blots were decorated with monoclonal antibodies against FRQ (αFRQm3G11) or against WC-1 (αWC-1m4H4) or affinity-purified rabbit antibodies against CK-1a. β-tubulin antibodies were a generous gift of Dr Manfred Schliwa. The FRQ antibody recognizes an epitope between residues 64 and 100 of FRQ and thus specifically detects the large form of FRQ. For αWC-1 a purified GST–WC-1 fusion (970–1071 amino acid residues) was used for immunization of mice. Western blots were performed using enhanced chemiluminescence (ECL) and ECL plus (Boehringer Mannheim). To control uniform loading of the gels,

nitrocellulose filters were stained with Ponceau S. In most cases cross-reacting bands appeared after longer exposures of the ECL blots, which were used to estimate equal loading of the gels. Similarly, the reactivities to β -tubulin and CK-1 α , which are expressed at constant levels, were used for normalization.

Immunoprecipitation and phosphatase treatment

For immunoprecipitation 1 ml of α FRQm3G11 or the indicated amounts of affinity-purified antibodies against CK-1 α were incubated with 20 μ l protein A–Sepharose beads (Pharmacia) in 1 \times TBS buffer for 60 min at room temperature. The beads were washed with TBS and 5 mg protein extract were added in a total volume of 500 μ l extraction buffer (50 mM HEPES pH 7.4, 10% glycerol, 137 mM NaCl, 5 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin A). Samples were incubated under agitation for 2 h to 10 h at 4°C. Protein A–Sepharose was sedimented and washed twice for 5 min with 1 ml extraction buffer. For dephosphorylation of FRQ the immunoprecipitate was incubated with λ -protein phosphatase (1000 U, New England Biolabs) for 30 min at 30°C in a total volume of 25 μ l. The reaction was stopped with SDS-sample buffer.

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